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Guanine Nucleotide and Magnesium Ion Regulation of the Interaction of Gonadotropic and β-Adrenergic Receptors with Their Hormones: A Comparative Study Using a Single Membrane System*

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ABSTRACT. The effects of guanine nucleotides and magnesium (Mg) on the interaction of catecholamines and gonadotropins with their receptors were studied in rabbit luteal membanes. The affinity of the luteal β-receptor for the agonists isoproterenol, epinephrine, and norepinephrine, as measured by inhibition of [^{125}I]iodohydroxybenzylpindolol ([^{125}I]IHYP) binding, was increased by 2 mM $MgCl_2$. The addition of 100 μM GTP or $MgCl_2$ in combination with GTP resulted in a reduction in the affinity of the β-receptor for these agonists. These effects of Mg and GTP were agonist specific, as the affinity of the luteal β-receptor for the antagonists propranolol and IHYP were not altered by Mg or GTP. The potencies with which isoproterenol, epinephrine, and norepinephrine competed with [^{125}I]IHYP for the luteal β-receptor and activated adenylyl cyclase activity indicated that the rabbit luteal adrenergic receptors were of the β_1 type. Comparison of the K_d values for these agonists calculated from binding experiments in the absence and presence of Mg, GTP, and combinations thereof with the K_{act} values (the concentrations of catecholamines required to stimulate adenylyl cyclase activity half-maximally) obtained in adenylyl cyclase assays demonstrated a positive correlation. This correlation was best when the K_{act} values were compared to the K_d values obtained in the presence of Mg plus GTP, suggesting that low affinity binding of catecholamines in the presence of guanine nucleotide is the biologically relevant affinity. In contrast, the affinity of the luteal gonadotropin receptor for hCG, human LH (hLH), ovine LH (oLH), and bovine LH (bLH) was not altered by GTP, as measured by direct Scatchard analysis of [^{125}I]iodo-hCG binding or inhibition of [^{125}I]iodo-hCG binding by hCG, hLH, oLH, or bLH. Mg slightly lowered the affinity of the receptor for [^{125}I] iodo-hCG, hCG, and hLH, while Mg increased the affinity of the receptor for oLH and bLH. However, in no instance did the addition of GTP alter the affinity of the receptor from that observed in the presence of Mg alone. A positive correlation existed between the K_d values calculated from receptor-binding experiments with K_{act} values obtained in adenylyl cyclase assays, suggesting that the specific gonadotropin-binding sites present in rabbit luteal membranes represent receptors which mediate the stimulatory effect of LH. Thus, it is concluded that the binding function of these two classes of hormone receptors are regulated differently. (*Endocrinology* **110:** 336, 1982)

GTP AND its analogs regulate adenylyl cyclase activity, which they stimulate, and the hormone affinity of a variety of adenylyl cyclase-coupled receptors, which they decrease. Among the receptors affected are both stimulatory and inhibitory receptors. Included in the former are liver glucagon receptors, adrenocortical angiotensin receptors, and turkey, rat, and frog β-adrenergic receptors; the latter include α_2-adrenergic receptors found in platelets and lung, opioid receptors in neuronal cells, and muscarinic acetylcholine receptors found in heart and central nervous system (for reviews, see Refs. 1 and 2). Studies with frog erythrocytes have shown that the magnitude of the decrease in affinity caused by GTP varies with the chemical nature of the ligand used, being roughly proportional to the intrinsic stimulatory (agonistic) capacity of the ligand (3). From these and other findings that include the fact that binding of competitive inhibitors (antagonists) is not affected by GTP or its analogs (4, 5), the concept has arisen that most, if not all, adenylyl cyclase-coupled receptors may be under the regulatory influence of guanine nucleotides and that a mechanistic relationship may exist between the decrease in ligand affinity and the stimulatory or inhibitory response elicited in adenylyl cyclase activity. However, LaBarbera *et al.* (6) and Amir-Zaltsman and Salomon (7) have reported that gonadotropin binding to corpus luteum and follicular membranes of the rat is not affected by guanine nucleotides. This raises the question of whether the effects of guanine nucleotides on receptor binding are indeed obligatorily related to the intrinsic mechanism by which receptors activate adenylyl cyclases

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or whether ovarian receptors are in some ways peculiar in that their mode of regulation might differ from that seen in other tissues. Since in previous experiments we had found that adenylyl cyclase activity in ovarian corpora lutea is stimulated not only by gonadotropins but also by catecholamines (8), the latter being a receptor shown in other tissues to be regulated by guanine nucleotides, and since we had also found that in rabbit corpora lutea both gonadotropins and catecholamines stimulate nonadditively a single adenylyl cyclase through a guanine nucleotide-dependent process that has characteristics similar to those expected on the basis of studies with other adenylyl cyclases (9), in this report we explore 1) whether gonadotropin interaction with rabbit luteal membranes is insensitive to guanine nucleotides, as shown in rat ovarian membranes, and 2) whether the catecholamine receptors in rabbit corpus luteum membranes show characteristics resembling those of other catecholamine receptors in other tissues or those of other (*e.g.* gonadotropin) receptors in the same tissue. The results obtained indicated that catecholamine receptor regulation in corpora lutea resembles that in other tissues, that fundamental differences exist in the mode of regulation of gonadotropin and catecholamine receptors, and that nucleotide effects on receptor binding are not a mechanistically indispensable feature in the nucleotide-dependent hormonal stimulation of adenylyl cyclases. A preliminary account of some of these results has been presented previously (10).

Materials and Methods

Materials

Inorganic ^{32}P was purchased from Union Carbide (Tuxedo, NY), ^{125}I (carrier-free) was obtained from Iso-Tex Diagnostics (Friendswood, TX), and [^{3}H]cAMP was purchased from Schwarz/Mann (Orangeburg, NY). ATP (Na-salt; catalog no. A-2383), GTP, cAMP, EDTA, Tris, creatine phosphate, and myokinase (2000 U/mg) were purchased from Sigma Chemical Co. (St. Louis, MO). Creatine phosphokinase (200 U/mg) was obtained from Calbiochem (LaJolla, CA). Highly purified hCG (hCG-CR119), ovine LH (oLH; NIH-LH-S19), bovine LH (bLH; NIH-LH-B9), and human LH (hLH; LER-960) were obtained from the NIH. (−)Isoproterenol, (−)epinephrine, and (−)norepinephrine were gifts from the Sterling-Wintrop Laboratories (Rennselear, NY). (−)Propranolol, (+)propranolol, and the hCG used to induce pseudopregnancy were gifts from Ayerst Laboratories (New York, NY). Hydroxybenzylpindolol (HYP) was a gift from Sandoz (East Hanover, NJ). All other chemicals and reagents were of the highest commercially available purity and were used without further purification.

[α-^{32}P]ATP (SA, >50 Ci/mmol) was synthesized according to the method of Walseth and Johnson (11) and purified by DEAE-Sephadex A-25 chromatography as described elsewhere (12). [α-^{32}P]ATP prepared by this method was supplied by the Core Laboratory on Cyclic Nucleotide Research, Center for Population Research and Studies on Reproductive Biology, Baylor College of Medicine (Houston, TX).

[^{125}I]Iodo-HYP ([^{125}I]IHYP) was prepared by iodination of HYP according to the method of Maguire *et al.* (13) and purified by high pressure liquid chromatography as described by Bearer *et al.* (14).

[^{125}I]Iodo-hCG was prepared using the lactoperoxidase procedure developed for LH (15) and adapted for hCG as follows. Iodination was carried out by mixing in the stated sequence 10 μl hCG (0.3 nmol), 5 μl 1 M phosphate buffer (K salt), pH 7.0, 5 μl carrier-free ^{125}I in 0.1 N NaOH (0.6 nmol; 1.32 mCi), 1 μl 1 mg/ml lactoperoxidase, and 2 μl 0.6 mM H_2O_2. After 1 min at room temperature, the reaction was stopped by the addition of 50 μl 1 mM sodium metabisulfite. Unreacted ^{125}I was separated from [^{125}I]iodo-hCG by gel filtration on Sephadex G-25. [^{125}I]Iodo-hCG prepared by this method had between 0.82–0.95 mol ^{125}I/mol hCG (SA, 40.9–46.5 μCi/μg; mol wt, 45,000) and showed biological potencies in adenylyl cyclase assays that were indistinguishable from those of uniodinated hCG (Fig. 1). Maximal bindability of the [^{125}I]iodo-hCG produced varied between 60–67% and was assessed by incubating for 90 min at 32.5 C, under the conditions described below for the [^{125}I]iodo-hCG binding assays, 5,000 and 10,000 cpm labeled material with 150 and 175 μg rat luteal membranes, prepared as described previously (8), and then determining the percent of added radioactivity bound. Under these conditions, the proportion of radioactivity bound did not vary by more than 1% (not shown).

Animals

New Zealand White rabbits (3.0–4.5 kg) were used throughout. Pseudopregnancy was induced by the injection of 100 IU hCG (Ayerst) in saline, iv. The rabbits were killed by cervical dislocation on day 7 of pseudopregnancy (the day of hCG injection was day 0). The ovaries were removed and placed in ice-cold Krebs-Ringer bicarbonate, pH 7.4, until dissection of the corpora lutea. The dissected corpora lutea were homogenized, and membrane particles were prepared as previously described (8).

Adenylyl cyclase assays

Adenylyl cyclase activity was determined at 32.5 C in medium containing 0.1 mM ATP (with 5×10^7 cpm [α-^{32}P]ATP), 0.6 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM cAMP (with 10,000 cpm [^{3}H]cAMP), 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, 0.02 mg/ml myokinse, and 25 mM Tris-HCl, pH 7.5. The standard incubation time was 10 min. The reaction was stopped, and the [^{32}P]cAMP formed was quantitated according to the procedures of Salomon *et al.* (16) as modified by Bockaert *et al.* (17).

[^{125}I]IHYP-binding assays

[^{125}I]IHYP-binding assays were carried out in the presence of 0.1 nM [^{125}I]IHYP (120,000 cpm), 25 mM Tris-HCl (pH 7.5), 1.0 mM EDTA, 0.1% bovine serum albumin, and 0.1 mM ascorbic acid in a volume of 500 μl. Incubations were performed for 30 min at 32.5 C. When present, the concentration of $MgCl_2$ was 2 mM and that of GTP was 100 μM. The reactions were stopped

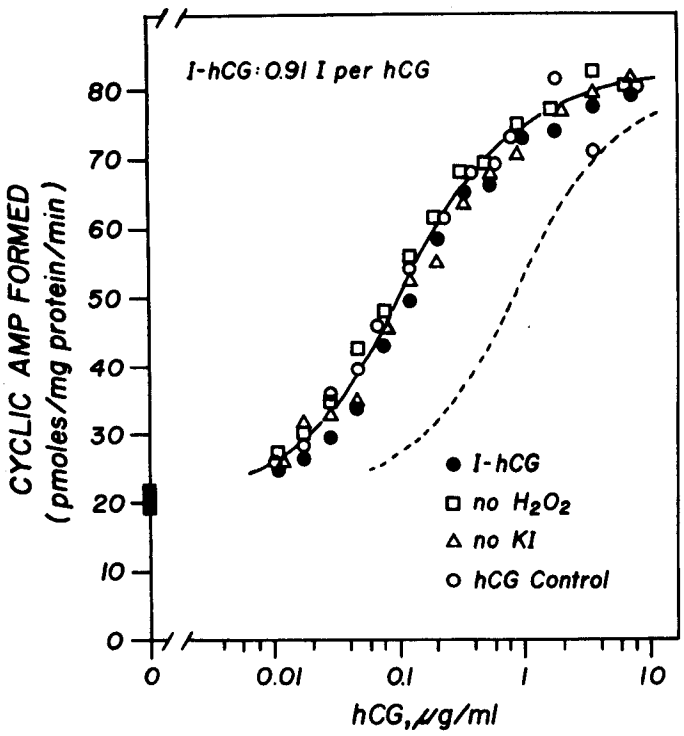

FIG. 1. Lack of effect of iodination and iodination-related manipulations on the biological potency of hCG. hCG was iodinated as indicated in *Materials and Methods,* except that 0.6 nmol KI with 100,000 cpm ^{125}I was substituted for the 1.32 mCi carrier-free ^{125}I (0.6 nmol). Bovine serum albumin was added to the stopped iodination mixture to give a final concentration of 0.1% in a final volume of 75 μl. This mixture was diluted serially, and aliquots were assayed directly for adenylyl cyclase-stimulating activity (●). The ability of this preparation to stimulate luteal adenylyl cyclase is compared to those of untreated hCG (○), hCG which had been subjected to the iodination reaction mixture in which H_2O_2 was omitted and H_2O was added instead (□), and hCG which had been subjected to the iodination reaction mixture in which the KI was omitted and replaced by an equal volume of 0.1 N NaOH (△). Adenylyl cyclase activity was determined as indicated in *Materials and Methods,* except that the concentration of ATP was 2.82 mM, that of $MgCl_2$ was 5 mM, and that of membrane protein was 12 μg/50-μl assay. Precipitation of an aliquot of the iodo-hCG (I-hCG)obtained with 15% trichloroacetic acid showed that 45.5% of the added ^{125}I had been incorporated into hCG. Based on this, it was calculated that the I-hCG was formed of a mixture of species iodinated to an average molar ratio of 0.91 mol iodine/mol hCG. - - -, Position of the concentration-effect curve if all iodinated species of I-hCG were biologically inactive. The data in this and other figures are representative experiments; each experiment was repeated with similar results at least two or three times.

by the addition of 1.5 ml ice-cold 2 mg/ml bovine γ-globulin in 0.1 M NaCl, followed by 0.5 ml ice-cold 20% polyethylene glycol (Carbowax 6000). The mixtures were left on ice for 10 min, centrifuged for 10 min in the cold at 3,000 rpm in a Sorvall RC-3 centrifuge (Ivan Sorvall, Inc., Norwalk, CT) affixed with an HL-8 rotor, and the supernatants were discarded by aspiration. The precipitates were resuspended by the addition of 1.5 ml 0.1 M NaCl and reprecipitated by the further addition of 0.5 ml 20% polyethylene glycol. The amount of [^{125}I]IHYP bound to the precipitates was determined after centrifugation and aspiration of the supernatant fluids. Nonspecific binding was measured in the presence of 10 μM (−)propranolol or 100 μM (−)isoproterenol. The values of bound [^{125}I]IHYP presented in the isotopic dilution curves correspond to the total bound without subtraction of reaction blanks of any kind. In some of the experiments, reaction blanks, *i.e.* radioactivities present in tubes incubated without membranes, were as high as 25% of maximal binding.

[^{125}I]Iodo-hCG-binding assays

[^{125}I]Iodo-hCG-binding assays were carried out in the presence of 5–1000 pM [^{125}I]iodo-hCG, 1.25% bovine serum albumin, 1.0 mM EDTA, and 25 mM Tris-HCl, pH 7.5, in a volume of 100 μl. Incubations were performed for 90 min at 32.5 C. When present, the concentration of $MgCl_2$ was 2mM and that of GTP was 100 μM. The reactions were stopped, and bound and free ligands were separated as described for [^{125}I]IHYP. Nonspecific binding was determined in the presence of 20 μg/ml oLH. The values of bound [^{125}I]iodo-hCG presented in the isotopic dilution curves correspond to the total bound without subtraction of reaction blanks of any kind. Reaction blanks, *i.e.* radioactivities present in tubes from which membranes had been omitted, were approximately 10% of maximal binding. At 50,000 cpm of added [^{125}I]iodo-hCG, specific binding of [^{125}I]iodo-hCG to membranes was linear with respect to protein up to 50 μg/assay.

Protein was determined by the method of Lowry *et al.* (18) using crystalline bovine serum albumin (fraction V) as standard.

The reported K_d values for the various unlabeled ligands (catecholamines and gonadotropins) were calculated from isotopic dilution curves according to the equation $K_d = (IC_{50}K^*)/K^* + [^*])$, where K^* is the K_d of either [^{125}I]IHYP or [^{125}I]iodo-hCG determined by Scatchard analysis, [*] is the concentration of [^{125}I]IHYP or [^{125}I]iodo-hCG used in the experiment, and IC_{50} is the concentration of unlabeled ligand causing 50% inhibition of specific binding of [^{125}I]IHYP or [^{125}I]iodo-hCG.

Expression of data

All data in the tables and figures are representative experiments; each experiment was repeated with similar results at least two or three times.

Results

We explored regulatory properties of receptor binding not only in terms of the effects of guanine nucleotide but also in terms of those of Mg ions. This was particularly indicated because 1) the stimulatory effects of both gonadotropin and catecholamine receptors on luteal adenylyl cyclase are critically and similarly dependent on the presence of Mg and guanine nucleotides (9), 2) Mg and guanine nucleotides have been shown to affect catecholamine binding in a variety of systems, including S49 cells and frog erythrocytes (19–22), and 3) the effects of both Mg and guanine nucleotides are absent in S49 cell lines that have a mutation in the regulatory component of adenylyl cyclase thought to be the molecule responsible for the regulation of receptor-binding functions (22).

Characteristics and regulation of catecholamine receptors

The binding of the antagonist [^{125}I]IHYP to rabbit luteal membranes was displaced stereospecifically by the β-adrenergic blockers (−) and (+)propranolol (Fig. 2). The concentration of (−)propranolol required to inhibit 0.1 nM [^{125}I]IHYP binding by 50% was 2 nM, compared to 450 nM for (+)propranolol. The α-adrenergic blocker phentolamine did not displace [^{125}I]IHYP (not shown). This indicated that the rabbit corpus luteum contains β-adrenergic receptors.

Scatchard analysis of [^{125}I]IHYP binding to rabbit luteal membranes indicated that these membranes contain between 110–140 fmol/mg membrane protein of specific binding sites which had a single affinity of 110 ± 15 pM (Fig. 3). The addition of 2 mM $MgCl_2$, 100 μM GTP, or combinations thereof neither altered significantly the affinity of [^{125}I]IHYP for the luteal β-receptor nor altered the total number of measurable β-receptors (Fig. 3).

The inhibition of [^{125}I]IHYP binding to rabbit luteal membranes by varying concentrations of (−)propranolol and (−)isoproterenol in the absence and presence of $MgCl_2$, GTP, and combinations thereof was measured (Fig. 4). The affinity with which the antagonistic β-receptor blocker (−)propranolol bound to rabbit luteal membranes was 0.9–1.1 nM. This affinity was not altered by guanine nucleotide or Mg. In contrast, the affinity of the stimulatory agonist (−)isoproterenol was profoundly altered by both Mg and GTP. It was found that in the absence of either Mg or GTP, isoproterenol bound to the luteal β-receptors with an affinity of 40–60 nM. The addition of 2 mM $MgCl_2$ resulted in a marked increase in the affinity of the receptor for (−)isoproterenol (K_d = 6–10 nM). On the other hand, the addition of 100 μM GTP resulted in a decrease in the affinity of the luteal β-receptor for (−)isoproterenol to K_d values between 160–240 nM, which were not significantly altered by the presence of Mg ion.

The order of potencies with which the various agonists isoproterenol, norepinephrine, and epinephrine displaced [^{125}I]IHYP binding (Fig. 5) indicated that the luteal receptor is not of the β_2 subtype and resembles that of the β_1 subtype. Isoproterenol demonstrated the highest affinity for the luteal β-receptor, followed by norepinephrine, which had an affinity that was only slightly different from that of epinephrine. This order of potencies was not changed by the addition of Mg, GTP, or combinations thereof (Table 1). As observed with isoproterenol, the addition of Mg resulted in an increase in the affinity of the luteal β-receptor for both norepinephrine and epi-

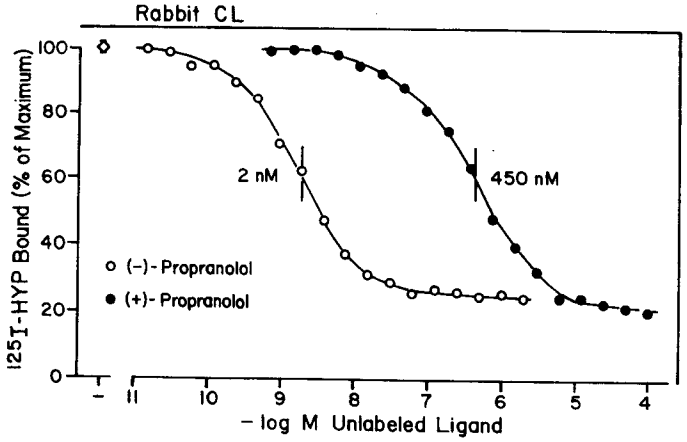

FIG. 2. Displacement of [^{125}I]IHYP binding to rabbit luteal β-adrenergic receptors by (−)propranolol and (+)propranolol. The vaues of [^{125}I]IHYP bound reported here correspond to the total bound without subtraction of reaction blanks of any kind. Incubations were carried out, as indicated in *Materials and Methods,* with 25 μg membrane protein/500-μl assay and the indicated concentrations of unlabeled (+) and (−)propranolol. Bound and free ligands were separated by the polyethylene glycol procedure described in *Materials and Methods.*

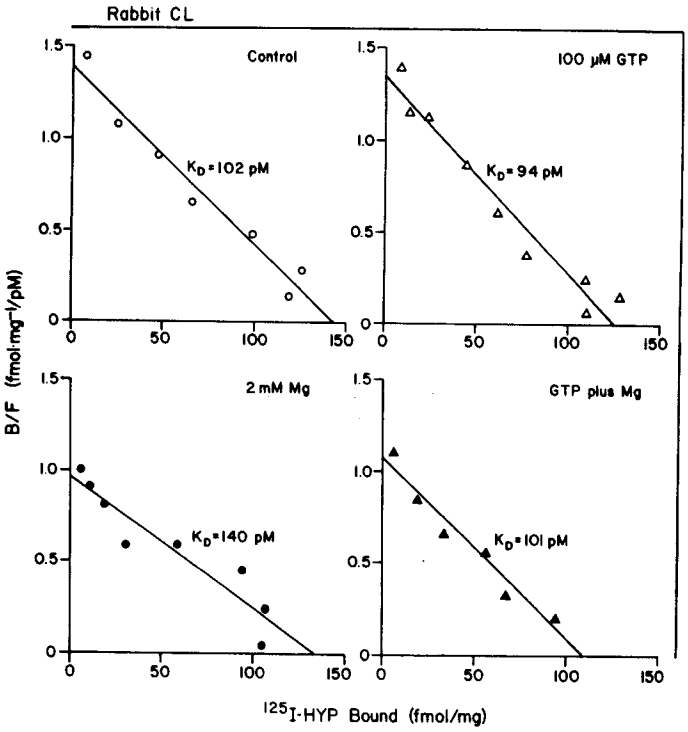

FIG. 3. Scatchard analysis of [^{125}I]IHYP binding to rabbit luteal membranes in the absence and presence of GTP and/or $MgCl_2$. Incubations were carried out, as indicated in *Materials and Methods,* in the presence of varying concentrations of [^{125}I]IHYP (0.02–4 nM). When present, the concentration of GTP was 100 μM and that of $MgCl_2$ was 2 mM. The membrane protein concentration was 25 μg/500-μl assay. All reactions were carried out in the absence and presence of 10 μM (−)propranolol. Specific binding was the difference between total [^{125}I]IHYP precipitated after incubation without excess (−)propranolol and that precipitated after incubation in the presence of excess (−)propranolol. Each *point* is the difference between the means of duplicate determinations. K_d values calculated from the slopes of the Scatchard plots are given.

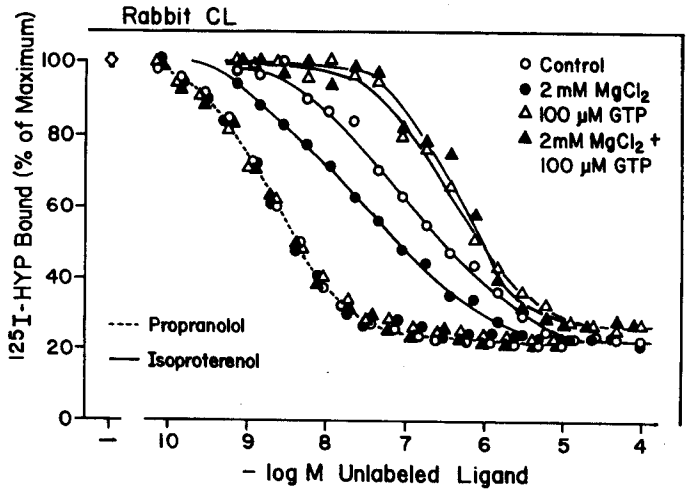

FIG. 4. Effects of GTP and Mg ion on isoproterenol binding and lack of effect of GTP and Mg and propranolol binding as seen in rabbit luteal membranes. The values of [^{125}I]IHYP bound reported here correspond to the total bound without subtraction of reaction blanks of any kind. Incubation conditions and determination of precipitable [^{125}I] IHYP by the polyethylene glycol precipitation technique were as stated in Fig. 2. When present, the concentration of GTP was 100 μM and that of $MgCl_2$ was 2 mM. The membrane protein concentration was 25 μg/500-μl assay.

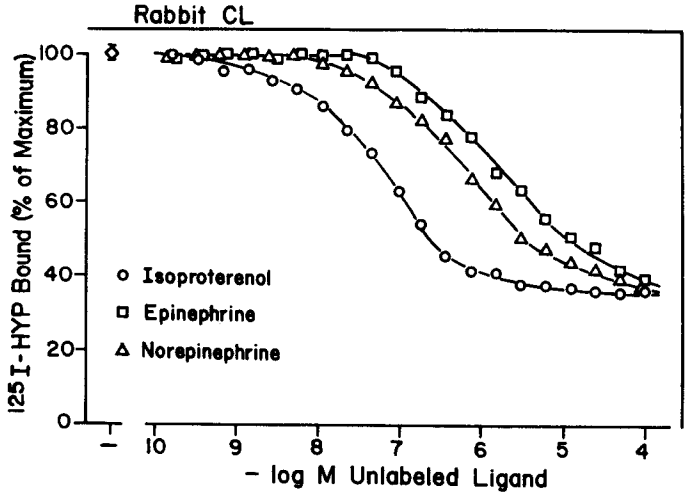

FIG. 5. Displacement of [^{125}I]IHYP binding to rabbit luteal β-adrenergic receptors by isoproterenol, epinephrine, and norepinephrine. The values of [^{125}I]IHYP bound reported here correspond to the total bound without substraction of reaction blanks of any kind. Incubations were carried out as described in Fig. 2, but in the presence of the indicated concentrations of unlabeled catecholamines and 0.1 mM ascorbic acid. The concentration of membrane protein was 25 μg/500-μl assay. For the rest of the conditions, see *Materials and Methods*.

nephrine, with the addition of GTP resulting in a decrease in the affinity of the luteal receptor for both norepinephrine and epinephrine to values that were not significantly altered by the further addition of Mg ion.

The potencies with which isoproterenol, epinephrine, and norepinephrine stimulate rabbit luteal adenylyl cyclase was also determined (Fig. 6). The order of potencies

TABLE 1. Effects of Mg and GTP on the abilities of isoproterenol, epinephrine, and norepinephrine to competitively inhibit the binding of [^{125}I]IHYP to rabbit luteal β-adrenergic receptor

Addition to binding assays	IC_{50} (nM)	K_d (nM)	Hill coefficient (slope of logit-log transformation)
A. Isoproterenol			
None	122 (101-146)	59 (49-70)	0.71 ± 0.03
$MgCl_2$	13 (7-28)	6 (3-13)	0.48 ± 0.07
GTP	486 (448-526)	234 (216-253)	0.88 ± 0.02
GTP + $MgCl_2$	290 (227-391)	140 (109-178)	0.85 ± 0.05
B. Epinephrine			
None	3855 (3481-4271)	1872 (1690-2073)	0.71 ± 0.02
$MgCl_2$	387 (313-478)	188 (152-232)	0.60 ± 0.03
GTP	6542 (5077-8430)	3176 (2464-4092)	0.89 ± 0.06
GTP + $MgCl_2$	4790 (3589-6394)	2325 (1742-3104)	0.85 ± 0.06
C. Norepinephrine			
None	689 (526-903)	331 (253-434)	0.66 ± 0.04
$MgCl_2$	238 (199-284)	114 (96-137)	0.60 ± 0.02
GTP	3075 (2506-3773)	1479 (1205-1814)	0.86 ± 0.04
GTP + $MgCl_2$	1966 (1449-2667)	945 (696-1282)	0.86 ± 0.06

Membranes were incubated with 0.1 nM [^{125}I]IHYP and varying concentrations of unlabeled catecholamines in the absence and presence of 2 mM $MgCl_2$, 100 μM GTP, and 100 μM GTP plus 2 mM $MgCl_2$, as described in *Materials and Methods*. The displacement curves obtained were linearized by the transformation: logit bound *vs.* log unlabeled catecholamine added. Logit bound = $\log[(bound_i - bound_{ns})/(bound_o - bound_i)]$, where $bound_i$ is the amount of [^{125}I]IHYP bound at a given concentration (i) of competing unlabeled hormone, $bound_o$ is the amount of [^{125}I]IHYP bound in the absence of added competitive hormone, and $bound_{ns}$ is the amount of [^{125}I]IHYP nonspecifically bound in the presence of excess unlabeled catecholamines. The intercepts with the x-axis provided the concentrations of unlabeled catecholamines giving 50% inhibition of binding (*i.e.* the IC_{50} values) in the form log IC_{50}. K_d values were calculated as indicated in *Materials and Methods*. The 95% confidence limits are in parentheses.

found was the same as that established in binding assays and indicated that catecholamines act through an adrenergic receptor of the β_1 subtype. Isoproterenol was more potent than norepinephrine, which was equipotent to epinephrine. Thus, it appears that both catecholamine-binding assays and catecholamine stimulation of luteal adenylyl cyclase evaluate properties of the same β-receptor. It should also be noted that all three catecholamines stimulated the rabbit luteal adenylyl cyclase to the same degree (Fig. 6), indicating that these catecholamines are full agonists in this system.

We also compared the various K_d values for isoproterenol, epinephrine, and norepinephrine in the absence and presence of Mg and/or GTP, calculated from isotopic dilution binding experiments (Table 1), to their K_{act} values (*i.e.* the concentrations of catecholamines required to stimulate adenylyl cyclase activity half-maximally) obtained in adenylyl cyclase experiments that were carried out under assay conditions that had been optimized for relative stimulation with respect to Mg ion and GTP (Table 2). The results obtained are presented in Fig. 7. It can be seen that there is a positive correlation between the K_d and K_{act} values. This correlation is best when K_d values obtained in the presence of $MgCl_2$ and GTP are compared to K_{act} values obtained in the adenylyl cyclase assay; the correlation is the poorest when K_d values obtained in the presence of $MgCl_2$ are compared to the

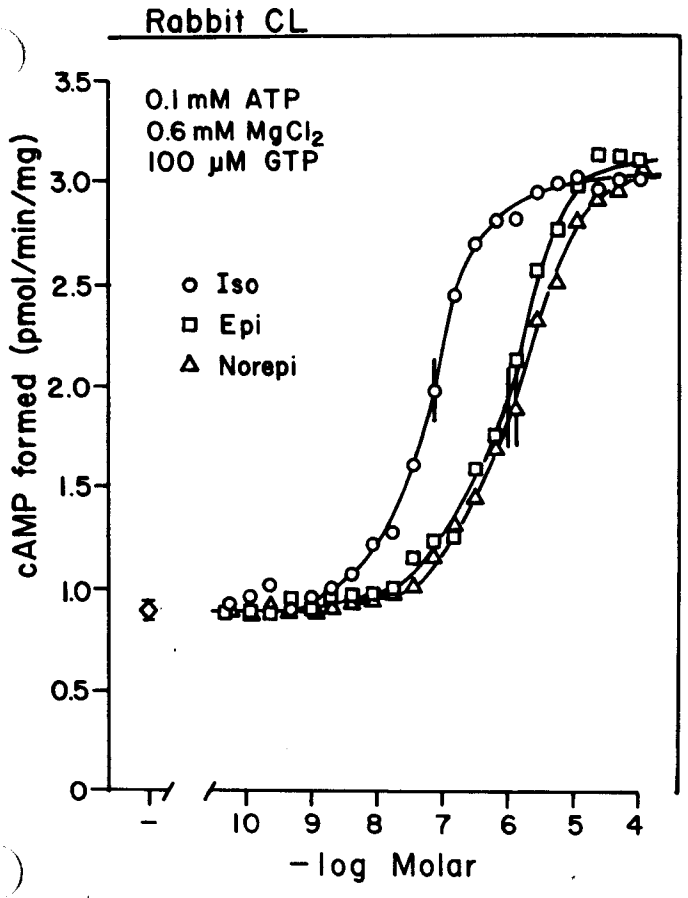

FIG. 6. Stimulation of rabbit luteal adenylyl cyclase by isoproterenol, epinephrine, and norepinephrine. Adenylyl cyclase activities were determined in the presence of 0.1 mM ATP, 0.6 mM $MgCl_2$, 0.1 mM ascorbic acid, and 100 μM GTP. The membrane protein concentration was 10 μg/50-μl assay. For the rest of the conditions, see *Materials and Methods*.

TABLE 2. Potencies of isoproterenol, epinephrine, and norepinephrine in stimulating adenylyl cyclase activity in rabbit corpus luteum membranes

Catecholamine	K_{act} (nM)	Hill coefficient (slope of logit-log transformation)
Isoproterenol	71 (54–94)	1.17 ± 0.10
Epinephrine	700 (417–1174)	0.90 ± 0.12
Norepinephrine	1199 (996–1442)	0.89 ± 0.04

Adenylyl cyclase activity was determined in luteal membranes incubated with varying concentrations of the indicated catecholamine, as indicated in *Materials and Methods*. The concentration-effect curves obtained were linearized by a logit activity *vs.* log added catecholamine transformation. Logit activity = log [(activity$_i$ − activity$_b$)/(activity$_m$ − activity$_i$)], where activity$_i$ is the activity in the presence of a given concentration (i) of hormone; activity$_b$ is the activity in the absence of hormone, and activity$_m$ is the activity in the presence of saturating concentrations of hormone. The intercepts with the x-axis provided the concentrations of hormone giving half-maximal stimulation (K_{act} values) in the form log K_{act}. The 95% confidence limits are in parentheses.

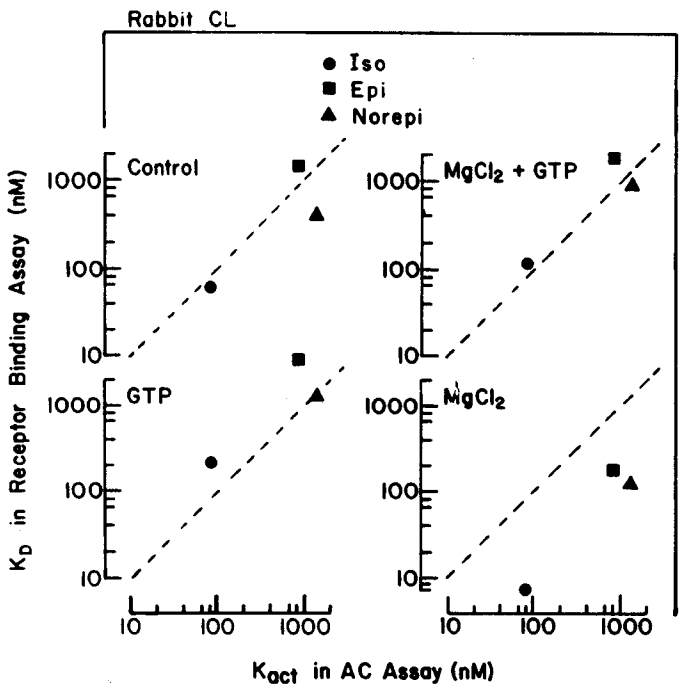

FIG. 7. Correlation of K_{act} and K_d values obtained for isoproterenol, epinephrine, and norepinephrine in adenylyl cyclase and receptor-binding assays, respectively. K_d were calculated from [^{125}I]IHYP displacement experiments in the absence and presence of $MgCl_2$ (2 mM), GTP (100 μM), and combinations thereof. – – –, Equivalence between K_d and K_{act} axes.

same K_{act} values.

Characteristics and regulation of gonadotropin receptors

Having determined that the interaction of β-receptors of rabbit luteal membranes with their stimulatory ligand are regulated by Mg and GTP in a manner similar to that seen for β-receptors in other systems such as S49 cells (19, 22) and frog and turkey erythrocytes (4, 5, 21, 22), we explored whether the regulation by Mg and guanine nucleotide was also evident for the LH receptors in these membranes, thus resembling other nonovarian nucleotide-and Mg-regulated systems, or whether it was absent, resembling rat luteal and follicular receptors (6, 7).

As reported before by others, we found rabbit luteal membranes to contain specific binding sites for hCG. Binding was relatively slow, requiring at 10 pM of total added [^{125}I]iodo-hCG approximately 90 min to reach a constant level. Increasing the concentration of [^{125}I]iodo-hCG decreased the time required to reach constant amounts bound (not shown), indicating that the rate of binding was concentration dependent. Using Scatchard's transformation of the binding data obtained after 90 min of incubation, linear plots were obtained which in the absence of Mg ion or guanine nucleotide yielded calculated K_d values of 40.8 ± 6.90 pM (mean ± SD of five

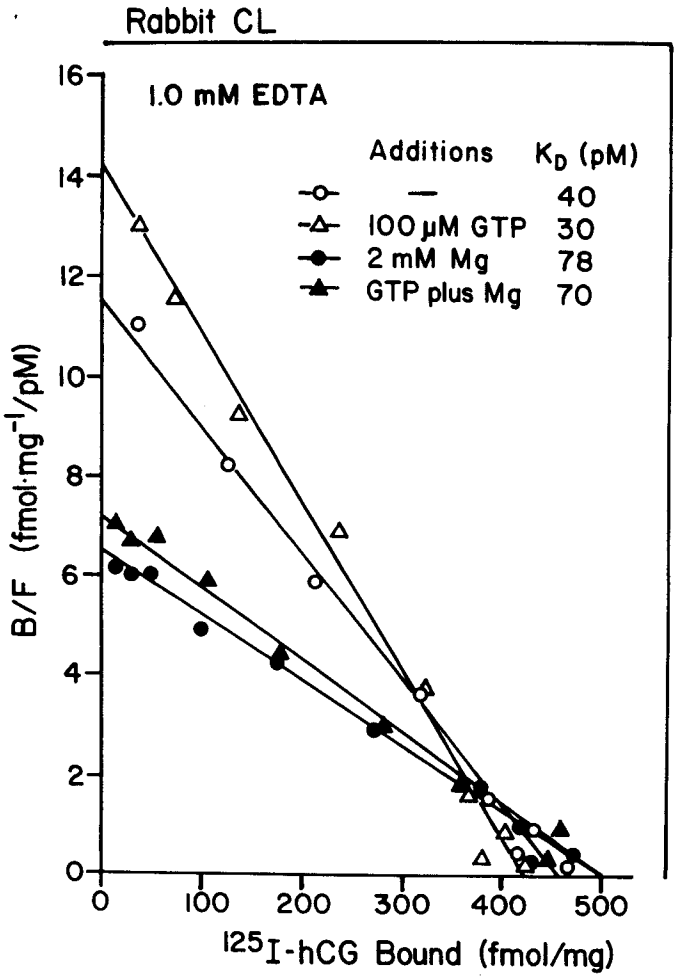

FIG. 8. Scatchard analysis of [^{125}I]iodo-hCG binding to rabbit luteal membranes in the absence and presence of GTP and/or $MgCl_2$. Incubations were carried out, as indicated in *Materials and Methods*, in the presence of varying concentrations of [^{125}I]iodo-hCG (0.87 mol ^{125}I/mol hCG). When present the concentration of GTP was 100 μM and that of $MgCl_2$ was 2 mM. The membrane protein concentration was 10 μg/100-μl assay. All reactions were carried out in the absence and presence of 20 μg/ml oLH. Specific binding was the difference between the total [^{125}I]iodo-hCG precipitated after incubation without excess oLH and that precipitated after incubation in the presence of excess oLH. Each *point* is the difference between the means of duplicate determinations. K_d values calculated from the slopes of the Scatchard plots are given.

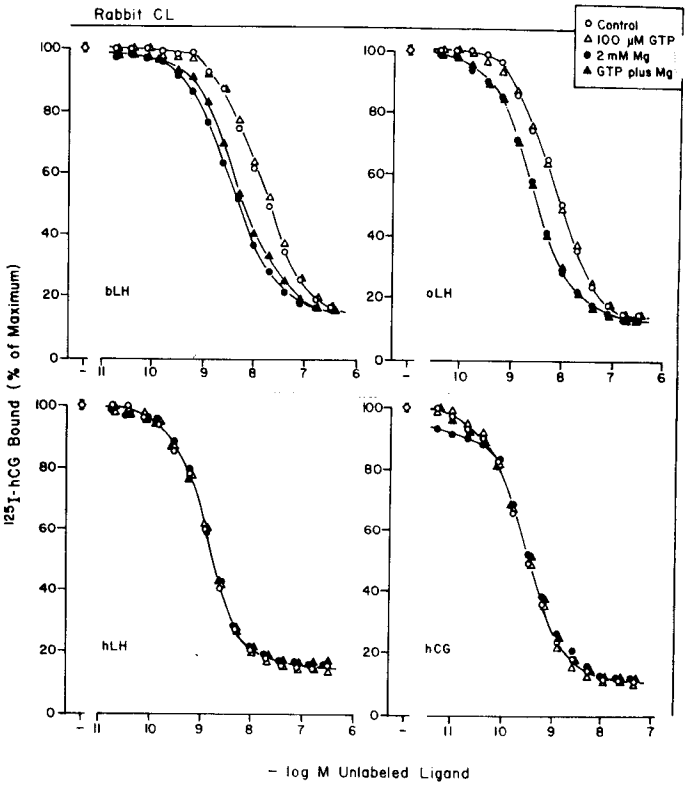

FIG. 9. Competitive inhibition of [^{125}I]iodo-hCG binding to rabbit luteal membranes by various LH preparations and hCG. Membranes (10 μg/100-μl assay) were incubated with 60–66 pM [^{125}I]iodo-hCG and the indicated concentrations of unlabeled gonadotropins in the absence and presence of 100 μM GTP, 2 mM $MgCl_2$, and combinations thereof. The values of [^{125}I]iodo-hCG bound shown correspond to the total [^{125}I]iodo-hCG precipitated at the end of 90-min incubations at 32.5 C by polyethylene glycol in the presence of 0.2% bovine γ-globulin without subtraction of any reaction blank.

determinations), with the total number of specific binding sites on rabbit luteal membranes being between 450–500 fmol/mg protein. The addition of $MgCl_2$, GTP, and combinations thereof led to the data shown in Fig. 8. In contrast to the findings with the catecholamines, the apparent affinity of the gonadotropin receptors for [^{125}I] iodo-hCG was not altered by guanine nucleotide; Mg had only a minimal effect, lowering the apparent affinity by a factor of about 2. This effect differed qualitatively from that seen with β-adrenergic agonists, the affinity of which were increased rather than decreased by Mg. Figure 9 illustrates that the lack of effect of GTP was not restricted to the binding of hCG, but was also observed with hLH, bLH, and oLH. On the other hand, Mg affected differently the binding of these gonadotropins to the rabbit luteal receptor. Calculation of apparent K_d values derived from the IC_{50} values obtained in the experiment illustrated in Fig. 9 (Table 3), indicated that while hCG and hLH were similarly affected (Mg caused a small and not statistically significant decrease in the calculated affinity constant), bLH and oLH appeared to increase their effectiveness in competing against [^{125}I] iodo-hCG when placed in the presence of Mg.

The abilities of various gonadotropin preparations to stimulated adenylyl cyclase activity under standard assay conditions are demonstrated in Fig. 10. Each gonadotropin preparation stimulated rabbit luteal adenylyl cyclase to a different degree. The greatest degree of stimulation was elicited by bLH, followed by oLH, which in turn stimulated adenylyl cyclase activity more than hLH. hLH and hCG elicited the same degree of stimulation from the rabbit enzyme. The potencies with which the various gonadotropin preparations stimulated luteal ad-

TABLE 3. Potencies of oLH, bLH, hLH, and hCG in competitively inhibiting the binding of [^{125}I]iodo-hCG to rabbit luteal gonadotropin receptors

Addition to binding assays	IC_{50} (nM)	K_d (nM)	Hill coefficient (slope of logit-log transformation)
A. oLH			
None	7.21 (6.48–8.03)	2.77 (2.49–3.08)	0.94 ± 0.04
GTP	7.62 (7.21–8.06)	2.83 (2.68–2.99)	0.98 ± 0.02
$MgCl_2$	2.55 (2.35–2.78)	1.33 (1.22–1.45)	1.14 ± 0.04
GTP + $MgCl_2$	2.80 (2.58–3.04)	1.45 (1.33–1.57)	1.02 ± 0.04
B. bLH			
None	12.5 (11.1–14.0)	4.61 (4.11–5.17)	1.03 ± 0.04
GTP	15.3 (12.6–18.6)	5.48 (4.50–6.66)	1.01 ± 0.06
$MgCl_2$	3.51 (3.33–3.71)	1.77 (1.68–1.88)	1.00 ± 0.02
GTP + $MgCl_2$	4.68 (3.81–5.75)	2.35 (1.91–2.88)	1.01 ± 0.07
C. hLH			
None	1.38 (1.16–1.65)	0.510 (0.429–0.610)	1.20 ± 0.07
GTP	1.46 (1.37–1.56)	0.522 (0.490–0.588)	1.21 ± 0.03
$MgCl_2$	1.37 (1.00–1.87)	0.692 (0.505–0.945)	1.21 ± 0.12
GTP + $MgCl_2$	1.44 (1.19–1.74)	0.723 (0.597–0.873)	1.34 ± 0.08
D. hCG			
None	0.270 (0.252–0.288)	0.103 (0.096–0.110)	1.15 ± 0.03
GTP	0.245 (0.228–0.261)	0.090 (0.084–0.097)	1.18 ± 0.04
$MgCl_2$	0.296 (0.272–0.321)	0.153 (0.141–0.166)	1.05 ± 0.03
GTP + $MgCl_2$	0.293 (0.281–0.307)	0.151 (0.145–0.158)	1.05 ± 0.02

Membranes were incubated with 60–70 pM [^{125}I]iodo-hCG and varying concentrations of unlabeled hormones [oLH (NIH-S19); bLH (NIH-B9); hLH (LER-960), and hCG (CR-119)]. K_d values for [^{125}I]iodo-hCG were calculated from Scatchard plots. These were (picomolar concentrations ± SD): 41 ± 7 (n = 5), 39 ± 9 (n = 3), 71 ± 9 (n = 2), and 70 ± 1 (n = 2) in the absence and presence of 100 μM GTP, 2 mM $MgCl_2$, and 100 μM GTP plus 2 mM $MgCl_2$, respectively. The displacement curves obtained were linearized by the transformation: logit bound *vs.* log unlabeled hormone added. Logit bound = log [(bound$_i$ − bound$_{ns}$)/(bound$_o$ − bound$_i$)], where bound$_i$ is the amount of [^{125}I]iodo-hCG bound at a given concentration (i) of competing unlabeled hormone, bound$_o$ is the amount of [^{125}I]iodo-hCG bound in the absence of added competitive hormone, and bound$_{ns}$ is the amount of [^{125}I]iodo-hCG nonspecifically bound in the presence of excess unlabeled hormone. The intercepts with the x-axis provided the concentrations of unlabeled hormone giving 50% inhibition of binding (*i.e.* the IC_{50} values) in the form of log IC_{50}. K_d values were calculated from the IC_{50} values, as described in *Materials and Methods*. The 95% confidence limits are in parentheses.

enylyl cyclase activity also varied; hCG was the most potent, while the potencies of hLH, oLH, and bLH did not differ significantly.

Since, as indicated above, [^{125}I]iodo-hCG binding to rabbit luteal membranes was slow, we wished to determine whether incubation of luteal membranes before the assessment of adenylyl cyclase activity would alter the K_{act} values for the various gonadotropin preparations. To this end, varying concentrations of each gonadotropin preparation were incubated in the presence of luteal membranes under adenylyl cyclase assay conditions without [α-^{32}P]ATP for 30 min, and adenylyl cyclase activity was monitored from 30–40 min by the addition of [α-^{32}P]ATP. It was found that preequilibration of receptors with gonadotropin resulted in a significant 4- to 7-fold reduction in the K_{act} values. This is illustrated for hCG in Fig. 11 and for the other gonadotropins in Table 4.

As with the catecholamines, we compared the apparent K_d values calculated from isotopic dilution binding experiments to the K_{act} values with which gonadotropins stimulate adenylyl cyclase activity. Two sets of K_{act}

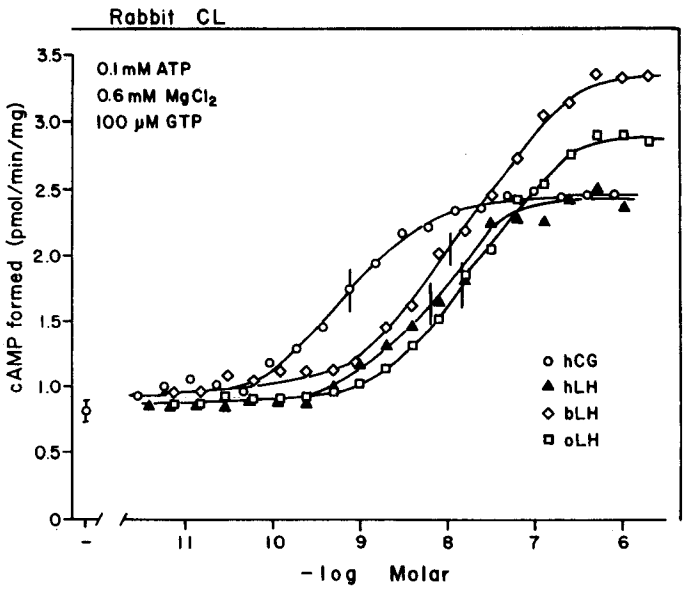

FIG. 10. Stimulation of rabbit luteal adenylyl cyclase by various LH preparations and hCG. Adenylyl cyclase activities were determined in the presence of 0.1 mM ATP, 0.6 mM $MgCl_2$, and 100 μM GTP. The membrane protein concentration was 10 μg/50-μl assay. For the rest of the conditions, see *Materials and Methods*.

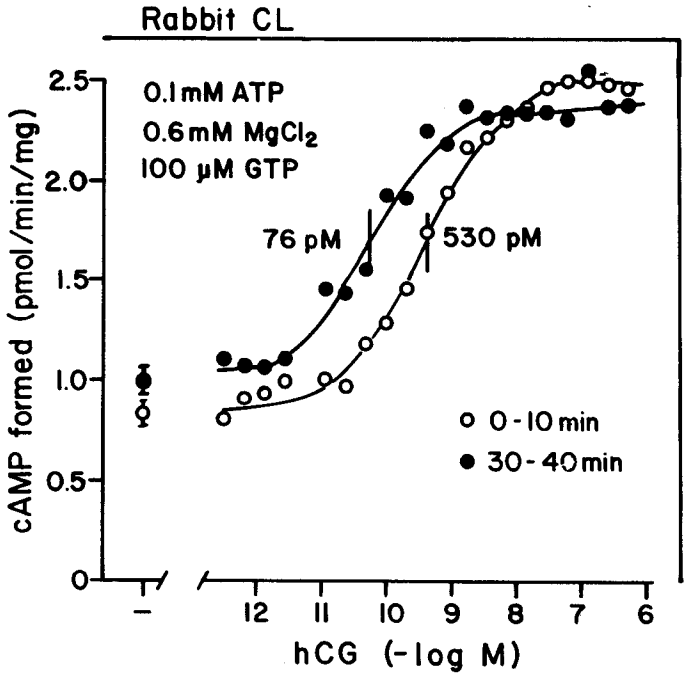

FIG. 11. Concentration-effect curves of hCG on rabbit luteal adenylyl cyclase as seen in 0- to 10-min assays and 30- to 40-min assays. The assay conditions are the same as those described for the experiments shown in Fig. 10.

values were used, one derived from standard 0- to 10-min assays and the other derived from 30- to 40-min assays. The results obtained are illustrated in Fig. 12. Clearly, there is a positive correlation between the K_d and K_{act} values. This correlation is strongest when K_d values

TABLE 4. Potencies of oLH, bLH, hLH, and hCG in stimulating adenylyl cyclase activity in rabbit corpus luteum membranes

Gonadotropin	K_{act} (nM)		Hill coefficient (slope of logit-log transformation)
A. 0- to 10-min assay			
oLH	18.1	(11.5–28.5)	0.89 ± 0.12
bLH	18.0	(12.1–26.8)	0.79 ± 0.09
hLH	12.3	(7.19–20.9)	0.83 ± 0.14
hCG	0.37	(0.29–0.47)	0.77 ± 0.04
B. 30- to 40-min assay			
oLH	3.71	(2.60–5.30)	0.98 ± 0.10
bLH	2.90	(1.79–4.70)	1.15 ± 0.17
hLH	2.82	(1.83–4.34)	0.85 ± 0.12
hCG	0.060	(0.029–0.176)	0.82 ± 0.16

Adenylyl cyclase activity was determined in luteal membranes incubated with varying concentrations of the indicated gonadotropins [oLH (NIH-S19), bLH (NIH-B9), hLH (LER-960), and hCG (CR-119)]. Assays were performed for 10 min under standard assay conditions or membranes were allowed to incubate in the presence of the gonadotropins for 30 min, followed by assessing adenylyl cyclase activity for 10 min (30- to 40-min assay), as described in *Materials and Methods*. The concentration-effect curves obtained were linearized by a logit activity *vs.* log added hormone transformation. Logit activity = $\log [(\text{activity}_i - \text{activity}_b)/(\text{activity}_m - \text{activity}_i)]$, where activity_i is the activity in the presence of a given concentration (i) of hormone, activity_b is the activity in the absence of hormone, and activity_m is the activity in the presence of saturating concentrations of hormone. The intercepts with the x-axis provides the concentration of hormone giving half maximal stimulation (K_{act} values) in the form log K_{act}. The 95% confidence limits are in parentheses.

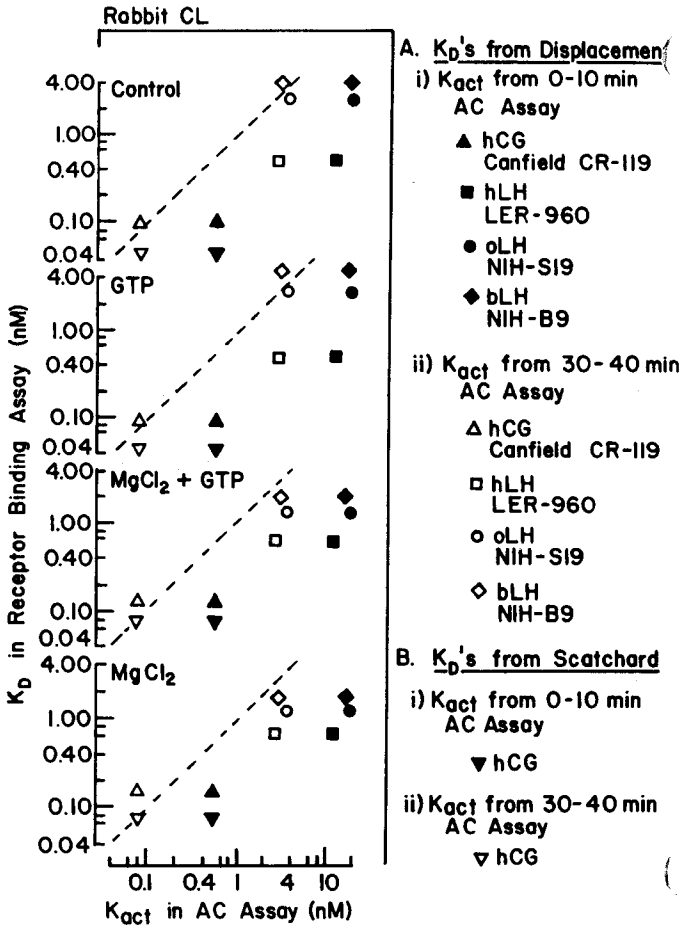

FIG. 12. Correlation of K_{act} and K_d values obtained for oLH, bLH, hLH, and hCG in adenylyl cyclase and receptor-binding assays. K_d values were obtained from experiments such as that shown in Fig. 9 in the absence (control) and presence of $MgCl_2$ (2 mM), GTP (100 μM), and combinations thereof. K_{act} values were obtained in 0- to 10-min assays (◆, ●, ■, ▲, and ▼) and 30- to 40-min assays (◇, ○, □, △, and ▽). ▽ and ▼, K_d values for hCG determined from Scatchard analysis. - - -, Equivalence between K_d and K_{act} axes.

obtained in the presence of GTP and Mg are compared to K_{act} values obtained in 30- to 40-min assays. The correlation between K_d values and K_{act} values would have probably been even greater if adenylyl cyclase activities were determined after a 90-min preequilibration, so that assessment of binding and activation of adenylyl cyclase activity were performed under identical conditions. However, this was impossible, since preincubations for periods longer than 30 min resulted in significant losses of both control basal activities and relative stimulations by the gonadotropins, possibly due to the thermal lability of both the transducing process and adenylyl cyclase itself. A similar correlation between [^{125}I]iodo-hCG binding and hCG activation of adenylyl cyclase has also been reported in rat follicular membranes (7). It appears, therefore, that the specific gonadotropin-binding sites present in rabbit luteal membranes represent receptors which mediate the stimulatory effect of LH.

Discussion

The effects of Mg on catecholamine binding were first reported by Williams *et al.* (21) and were found to be agonist specific. Bird and Maguire (22) discovered that these effects (increased affinity for receptor) were dependent upon the presence of an intact guanine nucleotide-binding regulatory component of adenylyl cyclase. However, the general pattern of Mg^{++} and guanine nucleotide action on agonist binding, as seen in displacement experiments similar to those reported here with a labeled antagonist as a receptor probe, appears to vary. In S49 cells (22, 23) and turkey erythrocytes (Iyengar, R., and J. Abramowitz, unpublished experiments), the potency with which isoproterenol displaces [^{125}I]IHYP in the absence of added Mg or guanine nucleotides is the same as when guanine nucleotides alone or Mg plus guanine nucleotides are added, and agonist affinity differs only when Mg alone is added, causing a leftward shift in the displacement curve. The experiments reported here with corpus luteum membranes show that the control curve differs not only from the displacement curve ob

tained in the presence of Mg (which is left-shifted), but so from the displacement curves obtained in the presence of guanine nucleotides. In still another Mg- and nucleotide-sensitive receptor system, that of the muscarinic adenylyl cyclase-coupled acetylcholine receptor from rat pituitary (24), receptor affinities for agonists are decreased progressively in the order Mg > control > GTP > GTP plus Mg. Since these effects appear to be mediated by the nucleotide-binding regulatory component of the adenylyl cyclase system, which is now known also to bind Mg ion (25), these differences are probably a reflection of differences in the regulatory properties of this component. However, at this time there is not enough information available to be able to interpret the meaning not only of the differences observed, but also of the mere fact that Mg and guanine nucleotides affect agonist binding to receptor.

We hoped to gain further insight into this puzzle by studying the effects of guanine nucleotides and Mg ion on gonadotropin binding. By exploring the effects of ligands on the two receptor systems in the same membrane, we were able to provide an internal control and

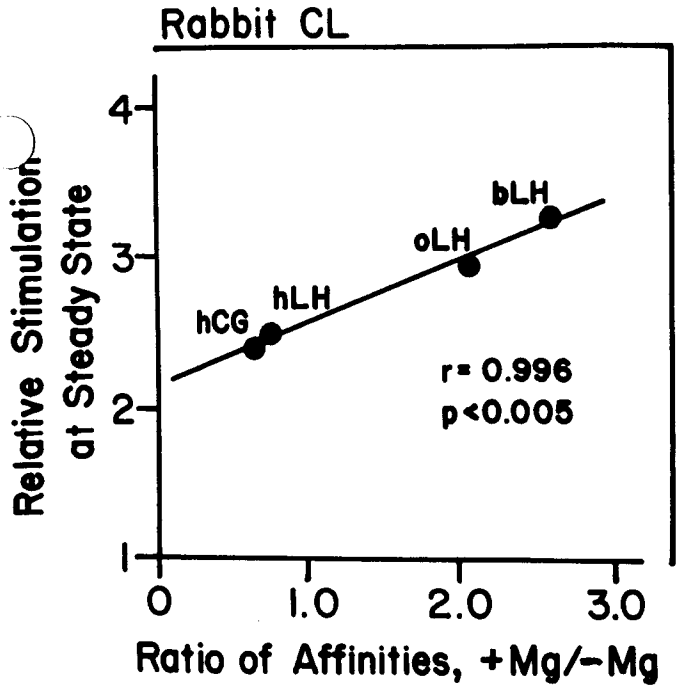

FIG. 13. Correlation of relative stimulation of adenylyl cyclase and ratio of affinities of the luteal gonadotropin receptor for oLH, bLH, hLH, and hCG obtained in the presence and absence of Mg. The relative stimulation of adenylyl cyclase by the various gonadotropin preparations was determined in experiments in which adenylyl cyclase activity was assessed from 30–40 min. The ratio of affinities of the receptor for the various gonadotropin preparations were determined from experiments such as that shown in Fig. 9 in the absence (control) and presence of 2 mM $MgCl_2$. Least squares linear regression analysis was used to determine the correlation between relative stimulation and the ratio of affinities.

corroborate the findings of Amir-Zaltsman and Salomon (7) and LaBarbera *et al.* (6) that gonadotropin binding is indeed unaffected by guanine nucleotides. This lack of effect is therefore neither species specific, since it is seen in both rats (6, 7) and rabbits as shown here, nor due to the presence of contaminating guanine nucleotides, for if this were so catecholamine binding should also have been seemingly insensitive to nucleotide addition. Since the effector system (*i.e.* the regulatory and catalytic components forming adenylyl cyclase) is common to both the gonadotropin and catecholamine receptors (9), and since regulation of catecholamine binding was readily observable, the lack of nucleotide effects on gonadotropin binding appears to be a consequence of one or more characteristics of the gonadotropin receptor itself.

Although insensitive to guanine nucleotides, gonadotropin binding was affected by Mg ion. As shown in Table 3, both the sense and the magnitude in the change in affinity obtained upon Mg addition varied with the gonadotropin used. Thus, Mg decreased the affinity of both hCG and hLH slightly by about 1.5-fold, a change opposite to that seen with catecholamines (21–23), glucagon (26), and a variety of other ligands, such as angiotensin (27), α-ligands (28), and muscarinic parasympathetic ligands (24, 29). On the other hand, the affinity of oLH was increased by 2.1-fold and that for bLH was increased by 2.6-fold. In view of the fact that the stimulatory effect of gonadotropins aligns them along the series hCG = hLH < oLH < bLH, the changes in receptor affinity for these gonadotropins upon Mg addition roughly correlates with the degree of stimulation they elicit (this is represented in Fig. 13). It is of interest to note that a similar type of correlation was reported by Lefkowitz and coworkers (3, 21) using 10 catecholamine analogs with varying capacities to stimulate adenylyl cyclase. Thus, the data at hand, although not conclusive, are at least consistent with the possibility that the capacity of Mg to induce changes in receptor affinity bears a relationship to the capacity of the affected hormone-receptor complex to stimulate adenylyl cyclase. However, the actual mechanism by which a Mg-induced shift in receptor affinity relates to the capacity of the receptor to activate the regulatory and catalytic components of adenylyl cyclase remains obscure.

Of interest for future studies and interpretation of data on the mechanism of activation of not only the luteal but also all adenylyl cyclases by hormone receptors, is the finding that nucleotides need not affect receptor binding for stimulation to occur. It should be noted that nucleotides did not affect the binding of gonadotropin in spite of the fact that under the conditions of incubation used, they were necessary for proper stimulation of the system by the gonadotropins (see Ref. 9). In view of the fact that the effect of Mg appears to be common to both types of

receptors, but that of nucleotides does not, it appears that future studies on the mechanism of hormonal regulation of adenylyl cyclases may benefit from paying more attention to the role(s) of the divalent cation. Studies are currently underway in this laboratory to explore the effects of hormonal stimulation on the interaction of Mg with both the regulatory and the catalytic components of the adenylyl cyclase system.

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